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Gene transcription and higher-level effects of multigenerational Zn exposure in *Daphnia magna*

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Abstract

Zn exposure of *Daphnia magna* during one generation has been shown to modulate gene transcription differently in Zn exposed organisms compared to their non-exposed offspring. Here we studied the transcriptional gene regulation with a cDNA microarray in *D. magna* exposed to Zn for three generations (F_0 - F_2). For the first time molecular effects of multigeneration toxicant exposure in *D. magna* are described. Out of 73 differentially transcribed genes in the F_1 Zn exposed generation (compared to the F_1 control), only 7 genes were also differentially transcribed in the same direction in the F_0 Zn exposed daphnids (up or down, compared to the F_0 control). The majority of the differentially transcribed unigenes in F_1 Zn exposed daphnids (78 %) were not differentially transcribed in the F_0 Zn exposed organisms. This indicates that Zn exposure affected other molecular pathways in the second exposed generation, although a reduced reproduction and a reduction in juvenile growth were observed in both Zn exposed generations, compared to the respective controls. In the third Zn exposed generation (F_2), no reduction in growth or reproduction compared to the control was observed. This acclimation was reflected in a significantly lower number of differentially transcribed genes, compared to the Zn exposed F_0 and F_1 generations.

Keywords

Acclimation, microarray, ecotoxicology, stress, ecotoxicogenomics

1. Introduction

In the young and rapidly growing research field of ecotoxicogenomics, genomic tools are used to detect the molecular responses an organism experiences when exposed to pollutants, providing clues to the toxic effects in the organism and the compensatory mechanisms that are induced (Poynton and Vulpe, 2009). With DNA microarrays, ecotoxicological effects of exposure can be linked with transcription profiles of large numbers of genes. The transcriptional patterns obtained provide a means to identify complex pathways and strategies that are altered or induced in an organism when it is exposed to environmental stressors (Steinberg et al., 2008). In recent years, a number of studies has investigated the transcriptional responses of *Daphnia* sp. exposed to different types of environmental stress, using *Daphnia* microarrays. This way, molecular effects induced by exposure of daphnids to e.g. Cd, dietary Zn, fenarimol, Ni and even binary metal mixtures or munitions constituents have been discovered and elucidated (Soetaert et al., 2007; Connon et al., 2008; De Schamphelaere et al., 2008; Garcia-Reyero et al., 2009; Vandenbrouck et al., 2009).

Under continuous, multigenerational exposure to certain metals, *Daphnia magna* is known to develop tolerance to this stress. This was demonstrated in experiments with Cd, Cu and Zn (Bossuyt and Janssen, 2004; Muyssen and Janssen, 2004; 2005). Molecular analyses can reveal insights into the underlying mechanisms of tolerance development during the acclimation period. This knowledge may be useful for screening or monitoring potential tolerance development in response to chemical exposure, or for investigating other environmental factors that could affect this tolerance. Except for an investigation of metallothionein induction, related to multigenerational Cd acclimation (Guan and Wang,

2006), no molecular studies related to tolerance development in metal acclimated *D. magna* are available in the literature.

In a recent study, transcriptional patterns of *D. magna* exposed to Zn for one generation and cultured under non-exposed standard conditions for two subsequent generations were analyzed using a custom cDNA microarray (Vandeghechuchte *et al.*, 2010b). This revealed transcriptional regulation of several genes, both in the exposed daphnids and in the two subsequent non-exposed generations. An interesting observation was that the differentially transcribed genes of the F₀ Zn exposed daphnids (compared to the F₀ control organisms) were different from those in their non-exposed F₁ and F₂ offspring (compared to F₁ and F₂ control daphnids).

In parallel with these two generations of non-exposed offspring, two generations of offspring were cultured under continuous Zn exposure. In the present study, gene transcription as well as higher-level effects in three generations of Zn exposed daphnids were studied to evaluate transcriptional effects of continuous multigeneration Zn exposure and to elucidate the acclimation process at a transcriptional level.

2. Materials and methods

2.1 *Daphnia* cultures and experimental design

D. magna Straus (clone K6) used in our experiments was originally collected from a pond in Kiel (Antwerp, Belgium) and has been successfully cultured under controlled laboratory conditions for more than 10 years in aerated carbon filtered tap-water, enriched with selenium (1 µg/L) and vitamins (7.5 mg/L thiamin, 100 µg/L cyanocobalamin and 75 µg/L biotin).

101 Daphnids were cultured in 10 mL medium per surviving daphnid during the first week and in
102 20 mL medium per surviving daphnid from the second week onwards, maintaining a
103 constant density of organisms and food, as described by Vandegehuchte et al. (2010b).
104 Culture media were renewed three times per week and juveniles were removed at these
105 occasions. The experimental design used in the current study is as follows. A set of neonates
106 (0-24h) taken from the laboratory culture was divided into two batches. One batch was
107 transferred to modified standard M4 medium (Elendt and Bias, 1990) and cultured in this
108 control medium for three generations (F_0C – F_2C). A second batch of neonates was transferred
109 into the same medium, but with the Zn concentration adjusted to 388 $\mu\text{g/L}$ and cultured in
110 this Zn contaminated medium for three generations ($F_0\text{Zn}$ – $F_2\text{Zn}$). Based on previous studies,
111 the higher Zn concentration was estimated to be sublethal, with a significant effect on
112 reproduction (Heijerick *et al.*, 2005; Muyssen and Janssen, 2005). Each combination of
113 generation and exposure (control or Zn contaminated medium) is termed a ‘treatment’
114 throughout this paper (Fig. 1). The standard M4 medium was modified by replacing EDTA
115 and Fe by 4 mg/L of natural Dissolved Organic Carbon (DOC) to avoid the use of excessively
116 high metal concentrations due to EDTA complexation and to increase the environmental
117 relevance of the medium. The dissolved organic matter was collected from a small unpolluted
118 creek (Ruisseau de St. Martin, Bihain, Belgium) using a portable reverse osmosis system (PROS/2)
119 (Sun *et al.*, 1995). It was stored in the dark at 4 °C in a 50 L barrel, at a concentration of
120 approximately 400 mg/L DOC. This DOC stock was thoroughly mixed each time before the
121 preparation of new medium. The same batch of DOC was used for all treatments and media
122 renewals. The Zn concentration in the control medium was adjusted to 19 $\mu\text{g/L}$ Zn, i.e. within
123 the optimal concentration range of this essential element for daphnids (Muyssen and
124 Janssen, 2004).

Reproduction as total number of living juveniles per surviving adult after 21 days was measured by counting the number of juveniles per organism three times per week for each individual daphnid. Ten individual daphnids were kept in plastic cages (fitted with 200 μ m mesh size gauze) which were suspended in the same aquaria as the treatment cultures. The length from the top of the head until the base of the spine was measured for ten different individual organisms per treatment by analyzing a microscopic image with UTHSCSA Image Tool 3.0 (San Antonio, TX, USA). This was done on day 6, day 13 and one to three days after the fifth brood was observed in the aquarium, when sufficient 0-24h offspring were available to start the next generation treatment. Internal Zn concentrations were determined as described in Vandeghechuchte et al. (2010b). All Zn concentrations were measured by atomic absorption spectrometry (SpectrAA-100, Varian, Mulgrave, Australia).

2.2 Statistical analysis

All statistics were performed with Statistica (Statistica, Tulsa, USA). Differences between the Zn exposed and the control daphnids in reproduction (total number of juveniles per surviving female), length or internal Zn concentration were assessed using t-tests. For the comparison of the internal Zn concentrations in daphnids from the three Zn exposed generations, a one-way ANOVA was used. Assumptions of normality and homoscedasticity were tested with Shapiro-Wilk's test and Bartlett's test, respectively. When one of these assumptions was not met, non-parametric Mann-Whitney U tests were performed to assess differences between exposed and control treatments (USEPA, 2000). In all tests, the limit of significance was set at $p = 0.05$.

2.3 Microarrays

Three *D. magna* cDNA libraries enriched with genes related to energy metabolism, molting and life stage specific processes have been developed by Soetaert et al. (2006; 2007) using the suppression subtractive hybridization technique. Next to these cDNA libraries, two extra cDNA fragments, corresponding to expressed sequence tags (ESTs) from genes that are reported to be sensitive to Zn were spotted on the array: ESTs with homology to (1) ferritin (AJ292556) and (2) retinol dehydratase (DV437801) gene fragments (Poynton et al., 2007). Finally, also two ESTs with homology to putative MTs (metallothioneins) (DV437799 and DV437826) were spotted because MTs have been shown to be induced by Zn (Fan et al., 2009). The preparation and spotting of the sequences are reported by Vandegehuchte et al. (2010b).

2.4 Microarray preparation

Three replicates of ten adult daphnids per treatment ('treatment' = combination of generation and exposure type, see Fig. 1) were sampled for mRNA analysis on the day the next generation was started (see above). The methods for RNA-extraction, conversion into cDNA, labeling and hybridization following a universal reference design can be found in Vandegehuchte et al. (2010b).

2.5 Bioinformatic analysis of microarray data

The microarrays were scanned using a Genepix personal 4100 Scanner (Axon instruments, USA). Scanned images were analyzed using Genepix Pro Software 4.0 (Axon Instruments) for spot identification and for quantification of the fluorescent signal intensities. Subsequently, data were further evaluated using the Bioarray Software Environment database (BASE

169 1.2.17, <http://www.islab.ua.ac.be/base/>), i.e. a MIAME based microarray analysis package
170 developed by the Intelligent Systems Laboratory (University of Antwerp, Belgium). Spots
171 were background corrected by local background subtraction. Spots with saturated intensities
172 were filtered out by visual inspection. The Cy5/Cy3 ratio was calculated for each spot, \log_2
173 transformed, and normalized between arrays using variance stabilization normalization
174 (Huber et al., 2002). Analysis of significant differences in transcription between treatments
175 was performed by using Limma (linear models for microarray data) (Smyth, 2004; Smyth et
176 al., 2005). Fragments for which the p-value, adjusted for false discovery rate, was lower than
177 0.05, were retained as significantly up- or downregulated (Benjamini and Hochberg, 1995).
178 Only those fragments for which the \log_2 ratio was outside the interval $[-0.75, 0.75]$ were
179 retained for further analysis. Sequence descriptions and annotations were obtained through
180 Blast2GO (Conesa et al., 2005)(www.blast2go.de), which allowed genes to be classified into
181 functional groups (Fig. 2). A heat plot was created with MultiExperiment Viewer (MeV) 4.5.1
182 (Saeed et al., 2006).

Results and discussion

Differences between exposed and control treatments will only be mentioned when they are statistically significant ($p < 0.05$).

An effect of Zn exposure on growth (vs. the respective controls) was noted in 6-day old daphnids of the F_0 Zn and F_1 Zn treatments (Fig. 3A, Table 1). Growth reduction in juvenile daphnids is not uncommon and has been observed in toxicity tests with cetyltrimethylammonium bromide and 5-azacytidine (Knops *et al.*, 2001; Vandeghechuchte *et al.*, 2010a). Like in the F_0 generation, a Zn induced reduction in juvenile growth (compared to the respective control) was also observed in their F_1 Zn offspring. However, no growth reduction was noted in the F_2 generation (compared to the F_2 control). The absence of growth reduction in the F_2 Zn daphnids can be interpreted as acclimation to Zn in the third exposed generation. This acclimation in the F_2 Zn organisms is also suggested by the fact that their reproduction is not affected (compared to the F_2 control daphnids), although reproduction results in F_2 should be interpreted with care, considering the decreased control reproduction in F_2 C. In the first and second generation of Zn exposed daphnids a reduction in reproduction was observed (compared to the control of the same generation, Fig. 3B, Table 1). Muysen *et al* (2005) showed that exposure to Zn for six generations can increase or decrease the reproductive output, depending on the acclimation concentration and the test concentration to which the sixth-generation daphnids were exposed. These authors reported a significantly higher reproduction in daphnids of the sixth versus the first generation acclimated to 45 $\mu\text{g/L Zn}^{2+}$ (which is higher than the optimal concentration range), when exposed to an optimal test concentration of 22 $\mu\text{g/L Zn}^{2+}$. In that study, reproduction in the actual acclimation treatments was not reported. Tolerance development/acclimation to a metal can occur even after two generations of exposure, as

207 demonstrated for net reproduction in *D. magna* exposed to 5 to 35 µg/L of Cu (Bossuyt and
208 Janssen, 2003). This is in accordance with our results on reproduction.

209 The average Zn body burdens of the exposed F₁Zn and F₂Zn treatments (resp. 165 and 157
210 µg Zn/g dry weight) were higher than those of the F₁C and F₂C controls (resp. 49 and 51 µg
211 Zn/g dry weight). This is in accordance with the previously reported internal Zn
212 concentrations of F₀Zn and F₀C (resp. 229 and 69 µg Zn/g dry weight, Vandeghehuchte *et al.*,
213 2010b). There was no significant difference between the internal Zn concentrations of the
214 three Zn exposed treatments.

215 When the gene transcription patterns of control treatments were compared (i.e. F₀C vs F₁C, F₁C vs
216 F₂C or F₀C vs F₂C), a large number of genes were found to be differentially transcribed, as reported by
217 Vandeghehuchte *et al.* (2010b). This concerned more than 15% of the unigenes on the array. The
218 differential transcription of these genes is likely due to differences in the molting phases and
219 reproductive cycles of the daphnids in the different generations and is as such not specific to the Zn
220 exposure. Therefore, those genes that significantly varied in transcription between different control
221 generations, were removed from the list of differentially transcribed genes between Zn treated
222 organisms and controls obtained with the microarray analysis. Thus, 38 to 46 % of the differentially
223 transcribed unigenes between treatments and controls were retained for further analysis. In the
224 following section of the manuscript, differential transcription will always be related to the
225 control of the same generation. Differentially transcribed genes for which a sequence
226 description could be obtained are listed in Fig. 2. Genes for which no homology was found
227 are summarized in the supplementary online material. Redundant fragments on the array
228 were grouped into contigs. The resulting 1207 unique identified fragments on the array are
229 termed unigenes (Vandeghehuchte *et al.*, 2010b).

230 In the F₁Zn daphnids, 73 differentially transcribed unigenes were found (Table 1). This
231 number is comparable to the 71 regulated unigenes in the F₀Zn treatment, where also a
232 reduction in reproduction and in body length at day 6 were observed. Seven genes were
233 regulated in the same direction in F₀Zn and in F₁Zn. However, another set of seven common
234 genes were differentially transcribed in opposite directions in F₀Zn and F₁Zn (Fig. 2 and
235 supplementary online table). Although some of the remaining 59 differentially transcribed
236 unigenes in F₁Zn may belong to the same gene as fragments that were differentially
237 transcribed in F₀Zn (such as genes with homology to *D. magna* vitellogenin or to a
238 hemoglobin subunit), for most of these fragments this is not the case. Zn exposure in the
239 second generation daphnids clearly elicited different effects at the transcriptional level
240 compared to the first generation. Some differentially transcribed genes in F₁Zn for which a
241 sequence description could be obtained through Blast will be discussed in the next
242 paragraphs.

243 General trends per functional group of genes differ between F₀Zn and F₁Zn organisms. While
244 in F₀Zn all affected transcription and translation related genes were downregulated, four out
245 of five transcription and translation related genes are upregulated in F₁Zn. All five of these
246 regulated unigenes are different from those in F₀Zn. The potential stress-induced energy-
247 saving mechanism of decreasing ribosomal protein synthesis (Brown-Peterson et al., 2005),
248 which was suggested based on the downregulation of ribosomal protein coding genes in
249 F₀Zn, is not present in the second generation of Zn exposed daphnids anymore. Similarly, the
250 oxidative stress response related genes peroxiredoxin 6 and glutathione S-transferase, which
251 were upregulated in F₀Zn, were not differentially regulated in F₁Zn.

252 While most metabolism-related differentially transcribed genes were upregulated in F₀Zn,
253 this was the case for only four out of nine metabolism-related differentially transcribed
254 genes in F₁Zn. A gene coding for a serine threonine protein phosphatase, which was
255 upregulated in F₀Zn, was downregulated in F₁Zn. In the presence of Fe²⁺, Zn²⁺ is known to
256 influence the activity of these phosphatases (Chu et al., 1996). It is hypothesized that in the
257 F₀Zn daphnids, the internally available Zn²⁺ concentration may have been high enough to
258 reduce the phosphatase activity compared to the control daphnids. A transcriptional
259 upregulation could compensate for this. Still following this hypothesis, the internally
260 available Zn²⁺ concentration may have changed in the F₁Zn daphnids, due to Zn induced
261 defense mechanisms, resulting in a phosphatase activity which is near the optimum and
262 higher than in the control, thus explaining the lower transcription. The upregulation of a
263 serine protease, as seen in the F₁Zn treatment, was also observed specifically after Zn
264 exposure in a study of transcriptional responses in *Daphnia magna* exposed to munitions
265 constituents, such as metals and nitroaromatic compounds (Garcia-Reyero et al., 2009).
266 Similarly, the observed downregulation of a chitinase is consistent with previous studies with
267 Zn exposed *D. magna*, where Zn toxicity was suggested to be associated with molting and
268 exoskeleton maintenance (Poynton et al., 2007; Garcia-Reyero et al., 2009).

269 The upregulation of a gene coding for the heat shock protein Hsp90 can be a stress response
270 leading to elevated levels of Hsp90 in Zn exposed daphnids, as observed in earthworms
271 exposed to Zn and Pb contaminated soils (Marino et al., 1999). Another likely stress
272 response, which was already noted in the F₀Zn treatment, is the upregulation of a gene
273 related to glutathione S-transferase, which is involved in oxidative stress abatement
274 (Newman and Clements, 2008). Also similar to F₀Zn, all differentially transcribed genes with
275 homology to *D. magna* vitellogenin, which is fused with a superoxide dismutase module

276 (Kato et al., 2004), were upregulated. These genes are involved with vitellogenesis, the
277 production of yolk proteins in the oocytes. Their differential transcription is likely due to
278 random differences in reproductive cycle phases and associated vitellogenesis between the
279 F₁Zn and F₁C daphnids, as indicated by the differential transcription between two control
280 treatments of a unigene with the same homology (Vandeghechuchte *et al.*, 2010b). Stibor
281 (2002) has demonstrated large differences in yolk protein levels at different times between
282 the deposition of two consecutive broods into the brood pouch.

283 The transcriptional downregulation of genes coding for a hemoglobin protein subunit was
284 already noted in F₀Zn. Martinez-Tabche et al. (2000) reported that Zn exposure decreased
285 the hemoglobin level in the oligochaete worm *Limnodrilus hoffmeisteri*. These authors
286 suggested that this was caused by a Zn induced inhibition of heme synthesis. If Zn inhibits
287 heme synthesis, it can be speculated that transcription of hemoglobin related genes would
288 not lead to the formation of hemoglobin protein and transcriptional downregulation could
289 be an energy-saving mechanism. Zn exposure is indeed known to decrease the hemoglobin
290 content in *D. magna* (Berglind, 1986). A last remarkable upregulated gene in the F₁Zn
291 treatment showed homology to cytochrome p450. P450s are proteins involved with phase I
292 detoxification, lipid metabolism and hormone synthesis/breakdown (Baldwin et al., 2009).
293 Transcriptional upregulation of a P450 coding gene in *D. magna* was also observed after Cd
294 exposure (Connon et al., 2008). Zn exposure, as well as Cu exposure, induced P450 activity in
295 earthworms (Lukkari et al., 2004).

296 It is striking that in the third generation of Zn exposed daphnids (F₂Zn) a much lower number
297 of genes than in the previous generations are differentially transcribed: only 23 of which 11
298 were upregulated (Table 1). Daphnids from this treatment seem to be acclimated to the Zn

exposure in the sense that no negative effects on reproduction or body length were observed, although the internal Zn concentration of 157 µg Zn/g dry weight in body tissue was still elevated and not significantly different from the previous Zn exposed generations. Roelofs et al. (2009) also reported a smaller number of Cd-induced differentially transcribed genes in a Cd tolerant versus a reference population of the springtail *Orchesella cincta*. Additionally, these authors suggested that the absence of inhibitory effects on translation and digestive enzyme related genes could explain the smaller growth reduction upon Cd exposure in tolerant *Orchesella* populations (Posthuma et al., 1992). Our results for Zn are in line with this suggestion. No growth reduction was observed in the ZnF₂ daphnids, for which only one translation and two metabolism related genes were differentially regulated, compared to six to seven and nine genes, respectively, in the previous generations with juvenile growth reduction. Two notable differences between the present study and that of Roelofs et al. (2009) can be remarked. First, springtails, unlike daphnids, are not parthenogenetic and thus genetic variation was present in their populations. Second, the springtails were selected from populations in different field sites, of which one had a long history of metal pollution, whereas the daphnids in the present study originated from the same parental generation and only differ in their three-generation exposure history. As such, no genetic selection can have acted on the daphnids in this study.

The genes for hydroxyisourate hydrolase (HIU hydrolase, involved in purine metabolism) and for obstructor d, involved in chitin metabolism, were downregulated in F₂Zn. Genes involved in chitin metabolism have been observed to be both up- and downregulated in several studies with metal exposed *D. magna* (Poynton et al., 2007; De Schamphelaere et al., 2008; Vandenbrouck et al., 2009). As in the other Zn exposed treatments, a gene coding for vitellogenin was upregulated and genes coding for hemoglobin subunits were

323 downregulated. Next to these, genes coding for a wd repeat protein and for a small
324 nucleolar ribonucleoprotein involved in mRNA splicing or its regulation as well as genes with
325 homology to chromosome 3 open reading frame 23 and to an inorganic pyrophosphatase
326 were downregulated. Transcriptional upregulation was observed for genes coding for two
327 proteins: one with homology to a hypothetical protein of the body louse *Pediculus humanus*
328 *corporis* and another one with homology to a midline fasciclin, which mediates cell adhesion
329 and signaling (Hu et al., 1998).

330 In conclusion, continuous Zn exposure resulted in acclimated *D. magna* in the third exposed
331 generation, which exhibited no adverse effect on reproduction or growth. At the
332 transcriptional level, few unigenes were regulated in the same direction in the three
333 generations of Zn exposed daphnids: two genes with no homology, a vitellogenin coding
334 gene and a hemoglobin chain coding gene. In the second Zn exposed generation (F₁Zn), a
335 large number of the differentially transcribed genes were different from those in F₀Zn,
336 although a reduction in reproduction and juvenile growth was observed in both treatments.
337 Multigenerational exposure to Zn elicits different molecular effects in the different
338 generations. The acclimation in the third exposed generation was reflected in a considerably
339 smaller number of differentially transcribed genes. No direct molecular acclimation
340 mechanisms could be deduced from the transcriptional results obtained with this custom
341 cDNA microarray, on which a limited, although ecotoxicologically relevant, set of genes is
342 represented. Currently, the *D. magna* genome is being sequenced by the *Daphnia* Genomics
343 Consortium, coordinated at Indiana University. When this genome becomes available, wider
344 transcriptome studies can be undertaken to elucidate the molecular mechanisms of metal
345 acclimation in *D. magna*.

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